

1 **Seeking to enhance the bioenergy of municipal sludge: Effect of alkali** 2 **pre-treatment and soluble organic matter supplementation**

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9 **Abstract**

10 The aim of this research is to enhance the mesophilic anaerobic digestion of municipal
11 sludge from Cadiz-San Fernando (Spain) wastewater treatment plant at 20 days
12 hydraulic retention time (HRT). Two different strategies were tested to improve the
13 process: co-digestion with the addition of soluble organic matter (1% v/v); and alkali
14 sludge pre-treatment (NaOH) prior to co-digestion with glycerine (1% v/v). Methane
15 production (MP) was substantially enhanced (from 0.36 ± 0.09 l CH₄ l/d to 0.85 ± 0.16 l
16 CH₄ l/d), as was specific methane production (SMP) (from 0.20 ± 0.05 l CH₄/g VS to
17 0.49 ± 0.09 l CH₄/g VS) when glycerine was added. The addition of glycerine does not
18 seem to affect sludge stability, the quality of the effluent in terms of pH and organic
19 matter content, i.e. volatile fatty acids (VFA), soluble organic matter and total volatile
20 solid, or process stability (VFA/Alkalinity ratio < 0.4). Alkali pre-treatment prior to co-
21 digestion resulted in a high increase in soluble organic loading rates (more than 20%)
22 and acidification yield (more than 50%). At 20 days HRT, however, it led to overload of
23 the system and total destabilization of the mesophilic anaerobic co-digestion of sewage
24 sludge and glycerine.

25 **Keywords:** anaerobic digestion; bioenergy, pre-treatment, mesophilic, sewage sludge,
26 glycerine

1. Introduction

Sludge treatment accounts for over 50% of the operating costs of wastewater treatment plants (WWTP) (Razaviarani and Buchanan, 2015; Rivero et al., 2014; Zahedi et al., 2017a). Anaerobic digestion (AD) is an attractive treatment strategy for municipal sludge and is of great benefit from an environmental point of view as this technology allow the production of bioenergy and fertilizer (Appels et al., 2011; Bolzonella et al., 2005; Di Maria et al., 2016, 2014; Forster-Carneiro et al., 2010; Gómez et al., 2006; Liao et al., 2016; Peces et al., 2016; Sosnowski et al., 2003; Wu et al., 2016; Zahedi et al., 2016a). It has been well demonstrated that mesophilic AD of municipal sludge from Cadiz allows the obtaining of Class B biosolids, i.e. an effluent with a density of faecal coliforms below 2×10^6 colonies g/1 total solids (Forster-Carneiro et al., 2010). Unlike Class A biosolids, which are essentially pathogen free and authorized for all uses, Class B biosolids may contain some pathogens and can be employed with a number of restrictions, such as crop harvesting, animal grazing, and public access for a certain period of time. Obtaining Class A biosolids requires an increase in temperature (thermophilic conditions, around 50 °C) (Riau et al., 2010). Numerous research studies have sought to optimize the AD of sludge, including the interesting options of the co-digestion process or sludge pre-treatments (Mata-Alvarez et al., 2011; Wang et al., 2013; Zahedi et al., 2016a), which increase the load of biodegradable organic matter and produce a higher biogas yield. The integrated management of sludge and fruit and vegetable waste by co-digestion and composting has recently been investigated from a life cycle perspective by Di Maria et al. (2016). Their results show that co-digestion enhances methane production. Recent studies have been also demonstrated the efficacy of anaerobic co-digestion of municipal sludge or solid waste together with readily

biodegradable organic substances, such as glycerol, a major by-product of biodiesel production (Fountoulakis et al., 2010; Fountoulakis and Manios, 2009; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015; Rivero et al., 2014; Zahedi et al., 2017c, 2016b). Studies on co-digestion have shown the optimal glycerine supplementation in the co-digestion of municipal sludge to be 1% (v/v) at 20 days hydraulic retention time (HRT) (Fountoulakis et al., 2010; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015). Due to slow sludge fermentation rates (hydrolysis and acidification) and the advantages of the anaerobic digestion (AD) process, extensive research has been carried out on the optimization of pre-treated sludge to improve hydrolysis, the generation of volatile fatty acids (VFA) and biogas production (Carrère et al., 2010; Ennouri et al., 2016; Lee et al., 2014; X. Li et al., 2016; Liu et al., 2012; Raynal et al., 1998). These pre-treatments seek to destroy cells and/or extracellular polymeric substances (EPS), with the subsequent release of intracellular and/or extracellular constituents to the aqueous phase (Carrère et al., 2010; Gianico et al., 2013; Wang et al., 2013). These released constituents are more easily biodegraded during anaerobic digestion, thereby enhancing methane production.

Most novel studies focus on combined methods, i.e. pre-treatment of substrates using different methods such as mechanical, chemical, thermal and/or others to increase their availability to microbial bioconversion (Dahunsi et al., 2016a).

One the most efficient, simple pre-treatments for municipal sludge is the alkali (NaOH) pre-treatment (Dahunsi et al., 2016a; C. Li et al., 2016; Zhang et al., 2015). For example, C. Li et al. (2016) reported that methane production in AD increased by 18% after microwave-ultrasonic pre-treatment or by 42% after pre-treating activated sludge

at 175 °C for 60 min or up to 71 % after pre-treating activated sludge at 120 °C with the addition of 20 mg NaOH

Taking into account the above, glycerine supplementation (1% v/v) and alkali pre-treatment in sludge was applied in the present research to improve the methane yield, achieving enhancements of between 71-125%. The experimental protocol was designed to examine the effect of two strategies for enhancing AD of the municipal sludge from Cadiz-San Fernando (Spain) WWTP. One was co-digestion of municipal solid sludge with glycerine (1% v/v), while the other was alkali sludge pre-treatment (NaOH) prior to co-digestion of municipal solid sludge with glycerine. It should be noted that this study was carried out at the most widely-employed hydraulic retention time (HRT) in the mesophilic AD of municipal sludge at most WWTP. Hence, the results of this paper provide useful information to obtain in-depth knowledge of strategies to enhance bioenergy production at WWTP.

To assess whether these strategies might be an interesting option in an actual municipal WWTP, different parameters such as the increase in SCOD (%), acidification yield (%), process stability, quality of the digested sludge and biogas production were studied.

2. Materials and Methods

2.1. Substrates, alkali pre-treatment and inoculum

Experimental work was carried out using sewage sludge samples (mixed primary sludge (30%) and activated sludge (70%)) from Cadiz-San Fernando WWTP. This plant is located in Cadiz-Spain and handles over 50,000 m³ of wastewater daily. All the sludge samples were characterized on reception at the laboratory and kept under refrigeration at 4 °C before being used for the experiments so as to prevent biodegradation. The pH,

volatile solids (VS) and soluble chemical oxygen demand (SCOD) concentrations in the municipal sludge were 6.8 ± 0.1 , 35 ± 2 g VS/kg and 10 ± 1 mg SCOD/l, respectively.

For the co-digestion studies (with or without alkali pre-treatment), this sludge was mixed with 1% v/v glycerol supplied by the Panreac company, which constituted the reactor feed. According to Fountoulakis et al. (2010), the most appropriate concentration of glycerol in co-digestion with sewage sludge in anaerobic processes is 1%.

For the alkali pre-treatment, the pH of the sludge sample was adjusted to 12.0 ± 0.1 , followed by stabilization for 5 min under stirring with 6 mol/L sodium hydroxide in line with Xiao et al. (2009).

Regarding the inoculum, this was collected from the mesophilic anaerobic digester (hydraulic retention time (HRT) = 20 d) located at the same WWTP. The pH, total solids (TS) and volatile solids (VS) were 7.5 ± 0.2 ; 32.0 ± 2.0 g TS/kg and 18.0 ± 0.2 g VS/kg, respectively. The inoculum to substrate ratio (ISR) in this reactor (g VS/g VS) was around 10.

2.2. Experimental equipment and operating conditions

Three laboratory-scale reactors operating in a laboratory-scale semi-continuous stirred tank reactor (CSTR) at the laboratory scale were employed in these studies. The reactors had a working volume of 5 l, without biomass recycling, and operated at the same HRT and Solids Retention Time (SRT), (20 days). Mesophilic conditions (35 °C) were maintained by circulating water through the jacket from thermostatic water baths.

PRECISTERM 6000142/6000389 (SELECTA S.A.) baths, with a maximum capacity of 7 l water, were used for this purpose, Mixing was maintained constant in the three

reactors using mechanical stirrers (23 rpm) and each reactor was equipped with a biogas outlet and a feed inlet. The gas volume produced in the reactor was emptied into Tedlar gas bags (40 l).

The three reactors employed were:

CR: fed with sewage sludge.

GR: fed with sewage sludge and glycerine (1%).AGR: fed with alkali pre-treated sewage sludge and glycerine (1%).

All the reactors operated at 20 days HRT (ISR around 10) and were fed once a day (semi-continuous) without the addition of nutrients or pH correction. The volatile solids organic loading rates (OLR) was 1.75 g VS/l/d.

The overall duration of the experiment for each reactor was 60 d, except in the AGR, where the overall duration was 7 days because destabilization was observed.

2.3. Analytical methods

The following variables were analysed to characterise and monitor the process effluents:

pH, alkalinity, volatile fatty acids (VFA), soluble chemical oxygen demand (SCOD) and volatile solids (VS). These analyses were conducted in accordance with standard methods (APHA, 1995) and Zahedi et al. (2017c). The gas volume produced in the reactors was measured directly using a high-precision flow gas meter: Ritter TG-01 drum-type gas meter - (wet-type).

VFA were determined by gas chromatography using a gas chromatograph (Shimadzu GC-2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol. The gas volume produced in the reactor was measured directly using a high-precision flow gas meter: Ritter TG-01 drum-type gas meter (wet-type). The composition of the biogas was determined by gas chromatography separation

(SHIMADZU GC- 2010). H₂, CH₄, CO₂, O₂ and N₂ were analysed by means of a thermal conductivity detector (TCD) using a Supelco Carboxen 1010 Plot column. Commercial mixtures of H₂, CH₄, CO₂, O₂, N₂ and H₂S (Abelló Linde S.A.) were used to calibrate the system.

Gas volume and composition were measured daily, as was the pH of the effluent. VS, COD, alkalinity and VFA were analysed approximately two/three times a week.

2.4. Parameters used to determine the effect of the different strategies on the reactor feeds

Changes in the soluble OLR (SOLR) and acidification yield were the parameters used to analyse the effect of the different strategies on the feed.

Acidification yield was calculated via the soluble COD of VFA (S_{TVFA}) through the following equation (De La Rubia et al., 2009; Zahedi et al., 2014, 2013):

$$\text{Acidification yield} = S_{\text{TVFA}} / S_s * 100 \quad (1)$$

where S_{TVFA} is the concentration of total VFA in the feed, expressed as mg COD/l using the theoretical COD equivalents for each VFA, and S_s is the soluble COD in the feed (mg COD/l).

3. Results and discussion

The effects of the different pre-treatments on the feed characteristics, effluent quality and the amount of biogas produced are assessed in this section.

3.1. Feed effect

Figure 1 shows the changes in the SOLR and acidification yield of the feed (municipal sludge) employed in each reactor. As can be seen, the SOLR was hardly affected by the different strategies. The SOLR of the municipal sludge was in the range of 0.50-0.6 g

SCOD/l/d- Logically, when glycerine (a soluble organic compound) was added to the sludge, the value of this parameter rose to 0.95 ± 0.2 g SCOD/l/d. When the sludge was previously alkali pre-treated, the value of this parameter increased from 0.95 ± 0.2 g SCOD/l/d to 1.15 ± 0.2 g SCOD/l/d. The increase in SOLR was due to the alkali pre-treatment destroying cells and/or extracellular polymeric substances (EPS) with the subsequent release of intracellular and/or extracellular constituents to the aqueous phase (Carrère et al., 2010; Gianico et al., 2013; Wang et al., 2013).

As to the acidification yield, the addition of glycerine to the substrate logically produces no differences in the acidification of the feed ($28 \pm 3\%$) (glycerine has COD, but is volatile fatty acid-free ; not all COD is due to VFA content). However, pre-treatment with NaOH produces a significant increase in the value of this parameter, from $28 \pm 3\%$ to $56 \pm 4\%$. These results were in line with those of other authors (Xiao and Liu, 2009; Zhang et al., 2015). The increase in VFA could be due to the degradation of lipids (Zhang et al., 2015).

The increase in SOLR was higher when alkali pre-treatment was also carried out. The main reason for this lies in the increasing pH value. Increasing the pH value changes cell osmotic pressure in sludge, resulting in EPS solubilisation and cell lysis (Zhang et al., 2015). Alkali pre-treatment also produces an increase in acidification yield. The increase in acidification yield could be due to the degradation of lipids. During NaOH pre-treatment, long chain fatty acids may be degraded and subsequently form low chain fatty acids. The highest mean values of organic matter solubilization and acidification yields in alkali pre-treated sludge supplemented with glycerine seem to suggest that this would be the ideal substrate for AD of sludge.

3.2. Process stability and effluent quality

The stability of the process was assessed via the evolution of pH and methane production (MP) in the each system (Rincón et al., 2008; Zahedi et al., 2017c). Figures 2 and 3 show the evolution of pH and MP, respectively, during the semi-continuous mesophilic study in each reactor. The reactors operated for 60 days, except for the AGR, as destabilization was observed after the first week. In Figure 2, a red horizontal line indicates pH 7.0. In the CR and GR, pH values stabilised around 7.3-7.8, the optimum pH for the activity of methanogenic microorganisms (Dahunsi et al., 2016b; Zahedi et al., 2017c). This means that a balance has been reached between the metabolic activities of microbial groups. However, even though the decrease in the effluent pH (<7.0) when NaOH (6 M) was added to the feed seems to be contradictory, it did take place. The pH dropped to values below 6 and the reactor did not recover, leading to a decrease in MP, due to the accumulation of VFA in the effluent. The acids generated during the acidogenic phase in the reactor were not completely consumed and accumulated in the system, thus affecting the activity of the anaerobic consortia, especially methanogens and acetogens, and leading to a reduction in methane production (Figure 3).

In the CR and GR, the total acidity, expressed as the total amount of VFA represented by acetic acid, alkalinity, VFA/Alkalinity ratio (equiv. acetic acid/equiv. CaCO_3) and TCOD, exhibited stable values in the effluent from the mesophilic reactors in the 120-655 mg acetic/l, 2-3 g CaCO_3 /l, 0.03-0.25 equiv. acetic acid/equiv. CaCO_3 and 7-11 g O_2 /l ranges, respectively. The VFA/Alkalinity ratio is a parameter used to assess the excess of overload in the substrate (Montañés et al., 2014; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015; Rincón et al., 2008; Zahedi et al., 2017c). Values

between 0.1 and 0.4 (equiv. acetic acid/equiv. CaCO_3) indicate favourable operating conditions without the risk of acidification. However, the values of VFA, VFA/Alkalinity ratio and TCOD in the AGR were respectively around 8000 mg acetic/l, 4 equiv. acetic acid/equiv. CaCO_3 and 17 g O_2 /l, while alkalinity value remained between 2-3 g CaCO_3 /l. This means the system was unstable or not suitable according to the decrease in pH values. The mean values of VFA and VS removal efficiencies (as %) for each reactor are shown in Figure 4. Removal efficiencies of around 47% VS removal and 82% VFA removal were obtained in the CR. The mean values of organic matter removal increased slightly in the GR. VS and VFA removal efficiencies increased from $47\pm6\%$ to $54\pm11\%$ and from $82\pm6\%$ to $89\pm2\%$, respectively. For the AGR, a huge decrease in organic matter consumption was detected, obtaining low VS removal efficiencies ($<13\%$) with an increase in VFA being observed, instead of VFA consumption. The increase in VFA content and the decrease in pH and organic matter removal in the AGR meant an overload in the system and non-stability in the effluent. Overload means loading an excessive amount of soluble substrate into the reactor. Overloading in a reactor produces intense COD solubilization and COD accumulation in the reactor due to kinetic decoupling between hydrolysis and methanogenic activities (Chen et al., 2012; Gianico et al., 2015). The acids generated during the acidogenic phase in the reactors were not completely consumed and accumulated in the system. In short, glycerine addition did not seem to affect the sludge effluent in terms of pH, organic matter content (VFA, SCOD and VS) or process stability (VFA/Alk ratios). However, mesophilic anaerobic digestion of the sewage sludge pre-treated with NaOH and supplemented with glycerine (1% v/v) (AGR) did not produce a stable effluent at 20 days HRT.

3.3. Biogas

The evolution of MP (l CH₄/l reactor/d), previously reported during the discussion of reactor stability, is shown in Figure 3. Figure 5 shows the mean values of MP (l CH₄/l reactor/d) and mean values of specific methane production (SMP, ml methane/ VS added) in each reactor. Mesophilic anaerobic digestion of sewage sludge (CR) produced mean values of MP ranging between 0.3-0.4 l CH₄/l/d and corresponded to values of SMP ranging between 0.15-0.25 l CH₄/g VS, respectively. When glycerine (1% v/v) was added to the feed (GR), a high increase in MP was observed (more than 120 %). Mean values of MP and SMP ranged between 0.7-1.0 l CH₄/l/d and 0.40-0.60 l CH₄/g VS, respectively. These results are in line with those of previous studies on municipal sludge and glycerine (Fountoulakis et al., 2010; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015). Fountoulakis et al. (2010) studied the feasibility of adding glycerol (1%) to anaerobic digesters treating sewage sludge. The reactor treating the sewage sludge produced 1106 ± 36 ml CH₄/d before the addition of glycerol and 2353 ± 94 ml CH₄/d after the addition of glycerol (1% v/v in the feed). Razaviarani et al. (Razaviarani et al., 2013; Razaviarani and Buchanan, 2015) studied the effect on process performance of adding increasing proportions of biodiesel waste glycerine to municipal wastewater sludge at 20 days HRT, reporting that methane production was 1.83 times greater than that obtained in their control digesters, which were only fed with municipal sludge. In the present study, glycerine addition (1% v/v) produced an increase in MP higher than 120 %.

The low values of SMP compared to those reported in other comparative papers (Fountoulakis et al., 2010; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015)

are due to the different municipal sludge employed. In the present paper, the sludge was mainly waste activated sludge (WAS) (around 70%), while in the studies by Razaviarani et al., the main waste was primary sludge (PS) (more than 70%). WAS has SMP values around 0.2 l CH₄/g VS (Wang et al., 2013; Zahedi et al., 2017b), whereas PS has SMP values ranging between 0.3 and 0.5 l CH₄/g VS (Peces et al., 2016; Razaviarani et al., 2013; Razaviarani and Buchanan, 2015; Zahedi et al., 2017a). The different origin of PS and WAS means they have different characteristics: WAS has a much higher content in microorganisms and proteins, but a lower fatty acids content and is less biodegradable. This means that it has a lower methane production potential than PS (Lens, 2004; Sato et al., 2001; Wilson and Novak, 2009; Zahedi et al., 2017a)

Anaerobic co-digestion of alkali pre-treated sludge did not lead to an increase in methane production. In fact, the decrease in MP was very considerable (lower than 0.12 l CH₄/l/d and 0.07 l CH₄/g VS), indicating, as already mentioned, that the pre-treatment does not improve biogas production efficiently. These results were due to the low pH and organic matter removals and the high VFA/Alkalinity ratio. The acids generated during the acidogenic phase in the reactor were not completely consumed and accumulated in the system, thus affecting the activity of the anaerobic consortia, especially methanogens and acetogens, and leading to a reduction in biogas production.

3.4 Optimal strategy to enhance AD at 20 days HRTAD of sewage sludge aimed at stabilizing the sludge and obtaining renewable energy was carried out under three different conditions (without any treatment, with the addition of glycerine, and with

alkali pre-treatment and glycerine addition). These supposed three SOLR (0.5, 0.95 and 1.15 SCOD/l/d) (Figure 1).

Alkali treatment was highly effective in terms of the solubilization parameters (Figure 1: SOLR and acidification yield). However, at least at 20 days HRT (the HRT employed in the actual digester at the Cadiz-San Fernando WWTP), the effluent was not found to be stable, nor was methane production seen to improve. This means that the efficiency of the single pre-treatment to improve the solubility of the waste and the effectiveness of the pre-treatment as regards methane production are not always correlated. There are other parameters that have to be considered, such as HRT, microbial activity, the OLR applied of the system, type of reactor, etc. This needs highlighting, as most pre-treatments are applied to the substrate, especially in secondary sludge, where methane production is often limited by the slow fermentation rates of this substrate (hydrolysis and acidification). Furthermore, many studies only focus on maximizing the increase in SCOD or VFA, or producing the greatest possible membrane damage in sludge cells. Sometimes, however, as in the present study, these changes do not necessarily lead to an increase in the biochemical methane of sludge. Zahedi et al. (2017a, 2017b, 2016a) also reported that a higher increase in SCOD, soluble proteins and damaged cells does not mean higher biodegradability or higher methane production. In fact, the most aggressive pre-treatment led to a higher increase in sludge solubilization and a decrease in SMP.

As regards the alkali pre-treatment plus co-digestion option, it may be stated that the addition of glycerine (1%) in the AD of municipal sludge could be an ideal strategy to improve the methane production at Cadiz-San Fernando WWTP, as the process was found to be totally stable, MP increased by around 120% and the quality of the effluent was not affected.

Two important overall conclusions can thus be drawn from this study. On the one hand, the addition of glycerine to municipal sludge from Cadiz-San Fernando WWTP at 20 days HRT considerably improved MP (120%) and could mean high economic benefits at a WWTP. This is an important fact, seeing as sludge management is a serious issue since up to one-half of the costs of operating WWTP is associated with sludge treatment and disposal (Lens et al., 2004; Peces et al., 2016; Zahedi et al., 2016a) and therefore any process that allows an increase in profits at the WWTP are worth highlighting. Furthermore, the efficiency of the single pre-treatment in improving the solubility of the waste and the effectiveness of the pre-treatment on methane production are not always correlated. As already stated, the most widely-used conditions for AD of municipal sludge at the majority of WWTP were employed in this study (mesophilic conditions (35°C) and 20 days HRT). Therefore, the results of this paper provide useful information for gaining in-depth knowledge of strategies to enhance bioenergy production at WWTP.

Conclusions

The effectiveness of the two strategies in improving AD of sewage sludge at 20 days HRT was assessed in this study. The following conclusions may be drawn.

Alkali pre-treatment was found to be the most successful means to increase sludge solubility. Under these conditions, the characteristics of the sludge were affected, significantly increasing the SOLR and acidification yield. However, at least at the HRT tested in the present study (20 days), this strategy alone was not effective and produced overload of the system (poor MP and effluent quality). The optimal conditions to

enhance MP were found under anaerobic co-digestion of municipal sludge and glycerine, resulting in an increase in MP of more than 120 % without altering the quality of the effluent in terms of the SCOD, VS, VFA, pH or VFA/Alkalinity ratio following digestion compared to the reactor fed without glycerine supplementation.

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Figure captions

Figure 1. Effect of the strategy on SOLR (g SCOD/l/d) and acidification yield (%)

values for each feed. CR feed (sewage sludge); GR feed (sewage sludge plus 1% v/v glycerine); AGR (feed: alkali pre-treated sewage sludge plus 1% v/v glycerine).

Figure 2. pH evolution (from 0 to 60 d) for each reactor: CR (feed: sewage sludge); GR

(feed: sewage sludge plus 1% v/v glycerine); AGR (feed: alkali pre-treated sewage sludge plus 1% v/v glycerine).

Figure 3. MP (ml CH₄/l/d) evolution (from 0 to 60 d) for each reactor: CR (feed:

sewage sludge); GR (feed: sewage sludge plus 1% v/v glycerine); AGR (feed: alkali pre-treated sewage sludge plus 1% v/v glycerine).

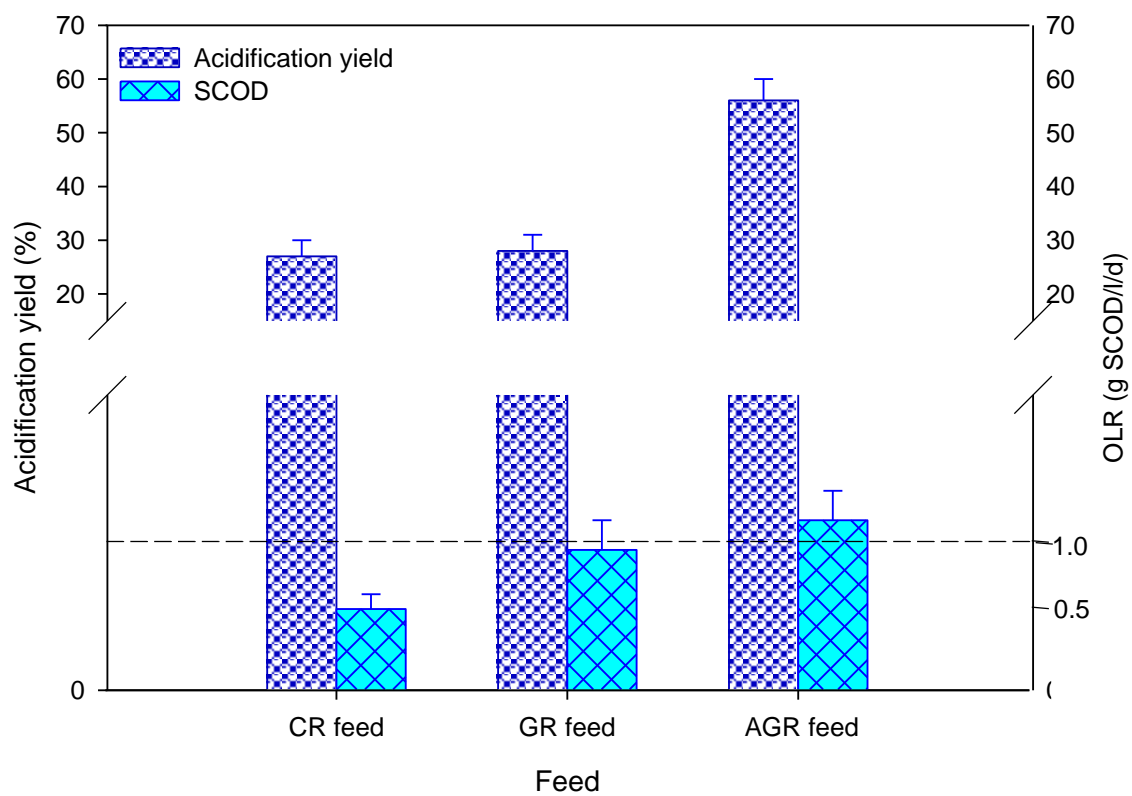
Figure 4. Mean organic matter removal values: VFA and VS removal for each reactor.

CR feed (sewage sludge); GR feed (sewage sludge plus 1% v/v glycerine); AGR feed (alkali pre-treated sewage sludge plus 1% v/v glycerine).

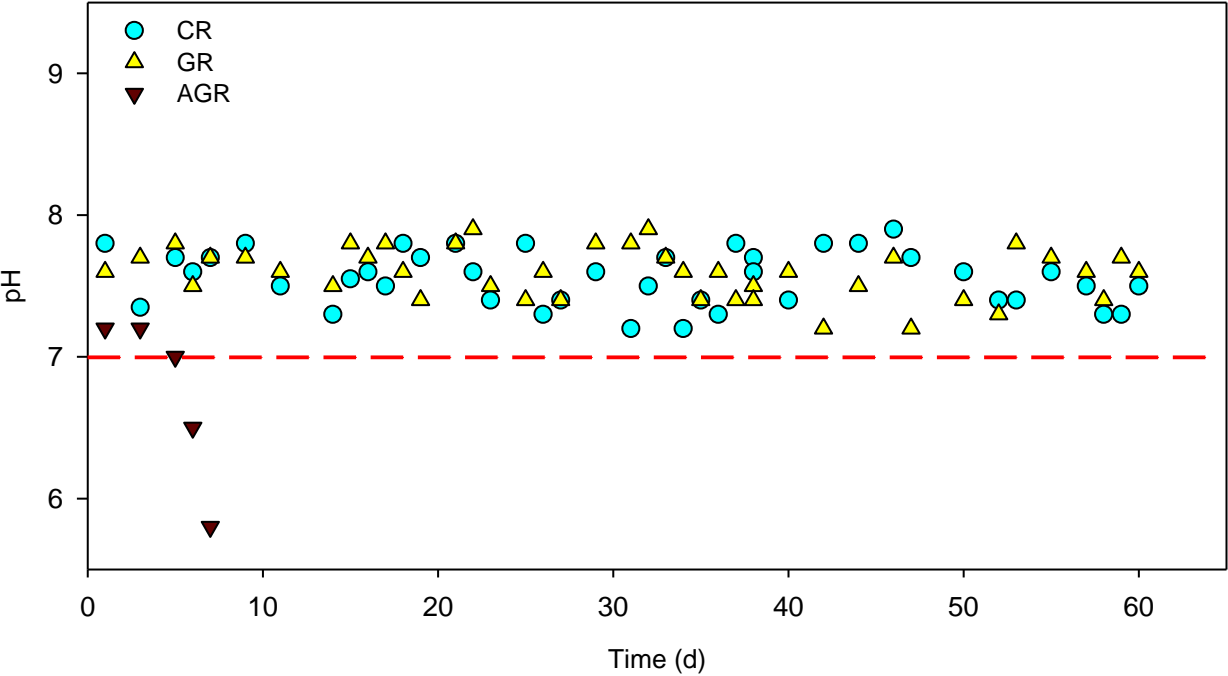
Figure 5. Mean MP (ml CH₄/l/d) and SMP (ml CH₄/ g VS) values for each reactor. CR

(feed: sewage sludge); GR (feed: sewage sludge plus 1% v/v glycerine); AGR (feed: alkali pre-treated sewage sludge plus 1% v/v glycerine).

Figure 1



539 **Figure 2**

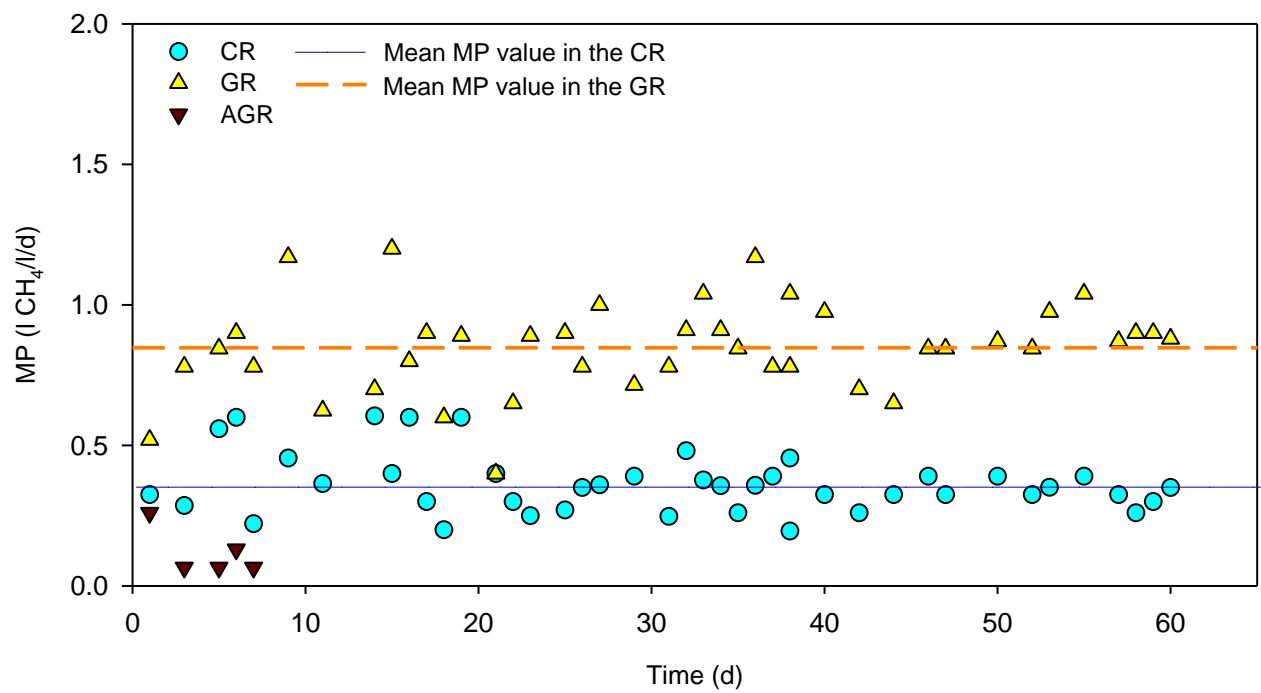


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542 **Figure 3**

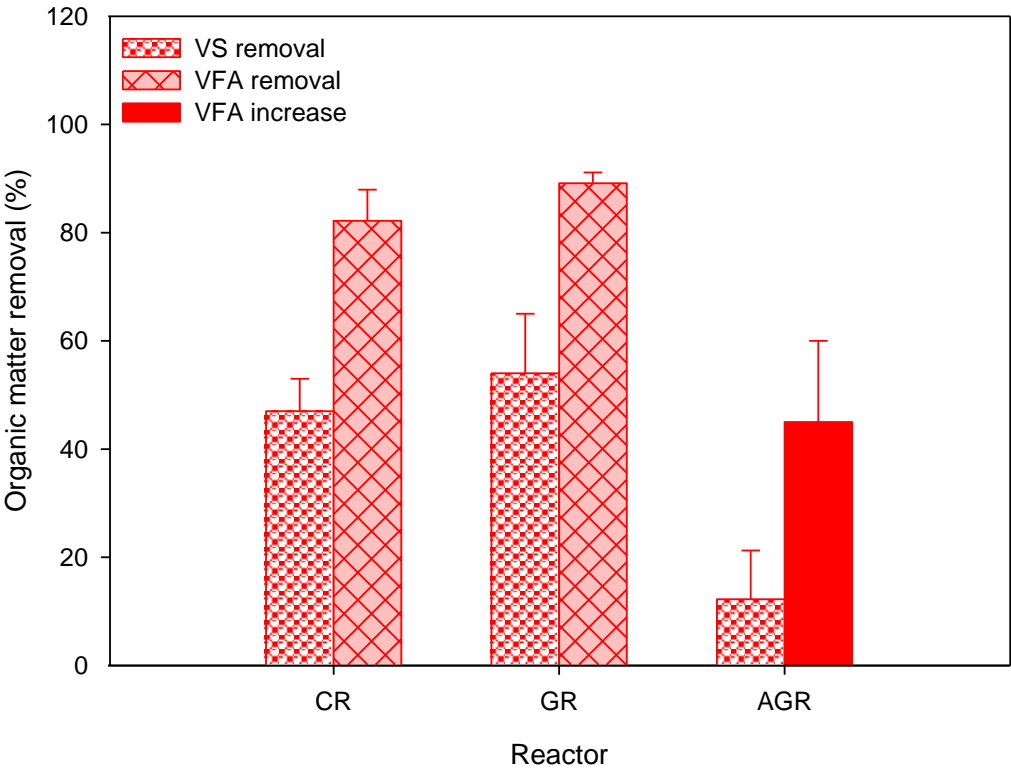
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546 **Figure 4**

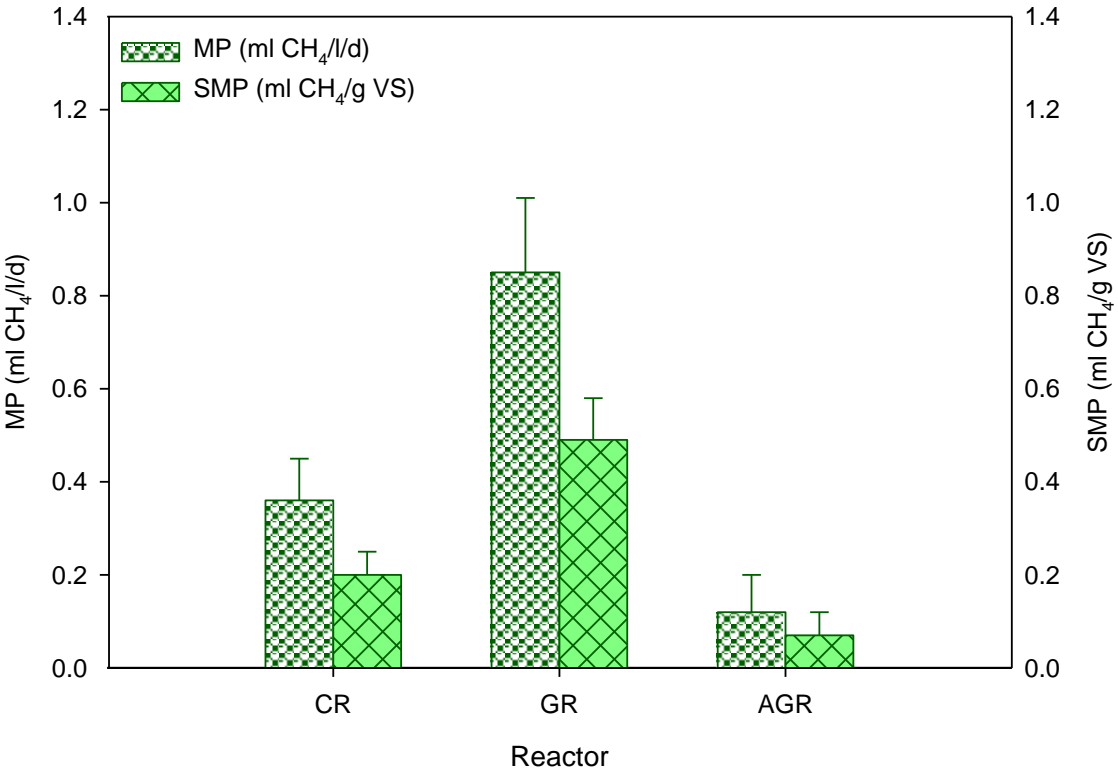


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550 **Figure 5**



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